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The role of an active transport mechanism in glycerol accumulation during osmoregulation by *Zygosaccharomyces rouxii*

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Summary. At water activities (a_w) of 0.998 (no osmoticum) and 0.960 a_w (NaCl), the affinity (K_m) of glycerol transport by *Zygosaccharomyces rouxii* was 25.6 and 6.4 mmol/l respectively. The maximum uptake rate (V_{max}) was ca. 2.3 μmol/g/min at both a_w's. However, at an a_w of 0.960 using polyethylene glycol (PEG) 400 the K_m and V_{max} for glycerol transport increased to 61.1 mmol/l and 32.2 μmol/g per minute respectively. This suggests that different glycerol transport mechanisms operate during stress by the two osmotica. The addition of uncouplers (2,4-dinitrophenol or carbonylcyanide-*m*-chlorophenylhydrazine) resulted in the outflow of accumulated [^{14}C]glycerol from *Z. rouxii* after an osmotic upshock indicating that an active transport mechanism was operative. The transport mechanism was specific for glycerol since other polyols (mannitol, *meso*-erythritol and arabitol) had no effect on the uptake rate. During upshock from 0.998 to 0.960 a_w (NaCl), a transient increase in the rate of [^{14}C]glycerol uptake was observed. However, if PEG 400 was used as osmoticum, the rate of glycerol uptake failed to increase.

Introduction

Cells respond to a decrease in water activity (a_w) by initially losing cellular water followed by the intracellular accumulation of solutes to equilibrate the cytoplasm osmotically with the surrounding medium (Brown 1978). Yeasts, including *Zygosaccharomyces rouxii*, accumulate glycerol as the primary osmoregulatory solute (Brown 1978; Reed et al. 1987). However, other polyhydroxy alcohols (polyols) such as arabitol, mannitol and *meso*-erythritol are also accumulated by some yeasts (Spencer and Spencer 1978) and may also act as osmoregulators (van Eck et al. 1989). These solutes are accumulated to high intracellular concentrations and glycerol concentration ratios (intra-/extracellular) greater than 1000-fold have been reported in yeasts such as *Z. rouxii* (van Zyl and Prior 1990), *Debaryomyces hansenii* (Adler et al. 1985) and *Hansenula anomala* (van Eck et al. 1989) when cultivated at decreased a_w (NaCl) values.

Z. rouxii responds to lowered a_w by retaining intracellularly a greater proportion of a constant amount of glycerol in batch culture (Edgley and Brown 1978) and continuous culture (van Zyl and Prior 1990). Therefore, *Z. rouxii* may be able to restrict the leakage of glycerol across the cell membrane or recover glycerol previously lost by diffusion when under osmotic stress. Changes in the composition and concentrations of fatty acids in osmotolerant yeasts grown at reduced a_w (NaCl) may decrease the membrane permeability (Tunblad-Johansson and Adler 1987; Watanabe and Takakuwa 1987). An active transport system able to retain a higher concentration of solute inside than outside the cell membrane may also be operative.

Little is known about the transport mechanism for acyclic polyols in yeasts. Yeasts such as *Saccharomyces cerevisiae* and *Candida utilis* take up glycerol by passive diffusion (Gancedo et al. 1968; Cooper 1982) while an active glycerol transport mechanism regulated by a_w (NaCl) has been found in *D. hansenii* (Adler et al. 1985). In this study we report on an active transport mechanism involved in glycerol accumulation by *Z. rouxii* under osmotic stress.

Materials and methods

Yeast strain. Z. rouxii (NRRL Y-998) was supplied by C. P. Kurtzman (Northern Regional Research Centre, Peoria, Ill, USA) and maintained on Yeast Malt (YM) slants (Wickerham 1951).

Media and growth conditions. A liquid medium consisting of minerals and vitamins (van Uden 1967) and containing 2% glucose as substrate was used. Vitamins and trace elements were filter-sterilized separately and added to the autoclaved medium. The a_w of the medium was adjusted to 0.960 with NaCl (1.20 molal; Robinson and Stokes 1959) or polyethylene glycol (PEG) 400 (BDH, Poole, Dorset, UK; 1.02 molal; Prior et al. 1977).

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Detection of proton symport. Cells for transport studies were cultured in 2-l erlenmeyer flasks containing 500 ml medium. The flasks were incubated at 30° C on a rotary shaker at 180 rpm. Growth was followed at 640 nm in an LKB Ultrospec II spectrophotometer (LKB Biochrom, Science Park, Milton Road, Cambs, UK). Dry mass was determined gravimetrically after the collection of cells by filtration. The presence of a proton symport in *Z. rouxii* was investigated by measuring the proton flux with a pH probe following the addition of glycerol to the cell suspensions as described previously (Lucas and van Uden 1986). Early exponential phase cells were harvested by centrifugation for 5 min at 15300 *g*, washed twice and resuspended in an isotonic solution of NaCl at 4° C. Starved cells were obtained by resuspending the harvested cells in a growth medium without carbon source and incubating the suspension at 30° C on an orbital shaker for 2 h. Thereafter, the cells were harvested, washed and resuspended as for non-starved cells.

Measurement of [^{14}C]glycerol uptake. Glycerol uptake was assayed in cells in the early exponential phase. Cells were resuspended in isotonic growth medium. Uptake of uniformly labelled [^{14}C]glycerol was initiated by the addition of 1.6 µl of dilutions of a stock solution of [^{14}C]glycerol (370 mmol/l) (Amersham International, Arlington Heights, Ill., USA; specific activity 4 GBq/mmol) to 50 µl cell suspension to final concentrations of between 3 and 31 mmol/l. The reaction was stopped after 1 min by the addition of 5 ml ice-cold isotonic NaCl solution (4° C). The samples were filtered (Whatman, Springfield Mill, Maidstone, Kent, UK; GF/C glass microfibre filters, 1 µm) and the cells washed with isotonic NaCl solution at 4° C. The filters were placed in scintillation vials containing 7 ml scintillation fluid (Aquagel I, Chemlab, Pinegrowie, Transvaal, RSA) and the radioactivity was determined with a liquid scintillation counter (LKB, Science Park, Cambs, UK; Rack Beta, Model 1217). Uptake was linear for at least 10 min.

Accumulation experiments. Accumulation experiments were carried out in 15 ml conical centrifuge tubes containing 0.03 g glucose. Cell suspensions (1.5 ml each) were added to the centrifuge tubes and osmotic upshock (0.998 to 0.980 a_w or 0.960 a_w) was initiated by the addition of NaCl or PEG 400. A stock solution (0.05 ml) of uniformly labelled [^{14}C]glycerol (370 mmol/l) was added to the suspension to obtain a concentration of 12.3 mmol/l [^{14}C]glycerol and samples were taken at appropriate time intervals. 2,4-Dinitrophenol (DNP; 2 mmol/l) or carbonylcyanide-*m*-chlorophenylhydrazine (CCCP; 5 µl of a 0.125 mmol/l stock solution) was added after 90 min to determine the effect of uncouplers on the accumulation of glycerol. The effect of NaCl concentration on the accumulation of glycerol at 0.980 a_w was investigated by preparing a series of cell suspensions containing increasing concentrations of NaCl. The water activity of each suspension was adjusted to 0.980 with sucrose (Robinson and Stokes 1959) using the equation: $a_w = a_w$ (NaCl) $\times$ a_w (sucrose). A stock solution of [^{14}C]glycerol was added to 650 µl cell suspension to a concentration of 25.6 mmol/l and samples were taken at appropriate time intervals.

Determination of the specificity of glycerol uptake. Cells grown in the medium adjusted to 0.960 a_w (NaCl) were harvested and prepared as described for the detection of proton symport. Arabitol, mannitol or *meso*-erythritol were added at concentrations 25 times greater than the glycerol concentration of 11.8 mmol/l and the uptake of [^{14}C]glycerol was measured as described above.

Determination of the rate of glycerol uptake during osmotic upshock. Osmotic upshock was induced by resuspending 1.5 ml harvested cells in medium adjusted to 0.980 or 0.960 a_w with either NaCl or PEG 400 in 15 ml conical centrifuge tubes. Samples (50 µl) were taken at appropriate time intervals. The rate of glycerol uptake was monitored by incubating 50 µl cell suspensions in the presence of 5.9 mmol/l [^{14}C]glycerol for 1 min and the accumulated glycerol concentration was determined as described above.

Results and discussion

Glycerol transport kinetics

Lineweaver-Burk and Eadie-Hofstee plots of the glycerol uptake rate (mean of four determinations with standard deviations in parentheses) by cells incubated at 0.998 a_w yielded a K_m of 25.6 (5.53) mmol/l and a V_{max} of 2.3 (0.34) µmol/min·g dry weight. Cell suspensions adjusted at 0.960 a_w (NaCl), yielded a K_m of 6.4 (0.76) mmol/l and a V_{max} of 2.4 (0.75) µmol/min·g dry weight. A K_m of 61.1 (7.32) mmol/l and a V_{max} of 32.2 (4.70) µmol/min·g dry weight were observed in *Z. rouxii* cells held at 0.960 a_w (PEG 400). The decrease in a_w from 0.998 to 0.960 a_w (NaCl) therefore did not affect the uptake rate significantly but increased the affinity of the transport mechanism for glycerol more than threefold. In *D. hansenii*, a_w had the opposite effect on the glycerol transport kinetics (Adler et al. 1985). A twofold increase in the V_{max} from 30 µmol/min·g dry weight (no osmoticum) to 61 µmol/min·g dry weight in 8% NaCl (ca. 0.950 a_w) but no change in the K_m (0.9 mmol/l) was reported. Furthermore the transport system in *D. hansenii* had a smaller K_m and a greater V_{max} for glycerol uptake than we observed in *Z. rouxii*.

D. hansenii is more tolerant to NaCl than glucose at equivalent a_w values whereas the opposite has been found in *Z. rouxii* (unpublished data) and this may be reflected in the properties of their transport systems. The difference observed in the kinetics of NaCl- and PEG 400-adjusted cells suggests that the different osmotica used to adjust the a_w regulate the glycerol transport differently. The K_m values for glycerol transport in *Z. rouxii* are greater than the average K_m values for active sugar transport but not as high as the K_m value for mannitol uptake by *S. cerevisiae* (0.6 mol/l) (Maxwell and Spoerl 1971).

The change in the kinetic properties of the glycerol transport system at decreased a_w may be related to an alteration in the membrane lipid composition and fluidity. Watanabe and Takakuwa (1984, 1987) reported that a decrease in the lipid unsaturation index of *Z. rouxii* corresponded to an increase in NaCl concentration. Prasad and Rose (1986) showed that the kinetic properties of various amino acid uptake systems of *S. cerevisiae* were affected by changes in the fatty-acyl unsaturation of the membrane. This suggests that a reduction in the fluidity of the cell membrane may affect the configuration of the transport proteins. Furthermore, the degree of fatty-acyl unsaturation and sterol content and composition of the cell membrane have been shown to affect monosaccharide transport in different yeasts (Prasad and Rose 1986).

Specificity of glycerol transport

The uptake rate of glycerol at 0.960 a_w (NaCl) was not significantly affected by the addition of unlabelled polyols to the cell suspension. In the presence of arabitol,

mannitol and *meso*-erythritol the glycerol uptake rates (mean of three determinations with standard deviations of the means given in brackets) of cells grown at 0.960 a_w (NaCl) were 90 (13), 99 (11) and 97 (14) % respectively of the cells incubated in the absence of the polyols. This suggests the presence of a highly specific glycerol transport mechanism in *Z. rouxii* similar to that observed in *D. hansenii* (Adler et al. 1985).

Accumulation of extracellular glycerol during osmotic upshock

When *Z. rouxii* cells were subjected to osmotic upshock from 0.998 a_w to 0.960 a_w, a rapid uptake of glycerol over 90 min was observed (Fig. 1). However, upshock with NaCl resulted in an equilibrium intracellular glycerol concentration approximately 50% greater than that observed when the cells were shocked with PEG 400. Considerably less glycerol was taken up by cells kept at 0.998 a_w than cells subjected to upshock. During upshock from 0.998 a_w to 0.960 a_w (NaCl), the ratio of the intracellular labelled glycerol to extracellular labelled glycerol increased from 0.24 to 4.26 (Table 1).

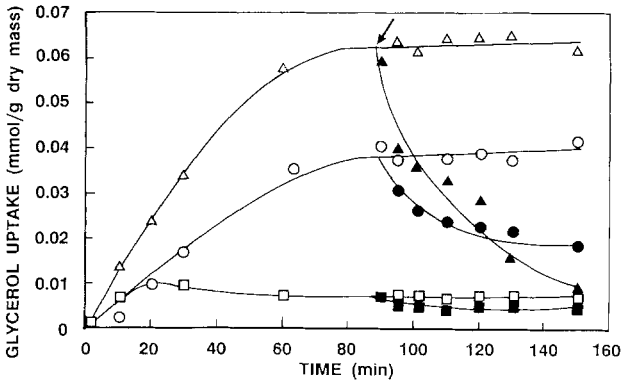

Fig. 1. Extracellular [^{14}C]glycerol uptake by *Zygosaccharomyces rouxii* in response to osmotic upshock from 0.998 water activity (a_w) to 0.960 a_w adjusted with NaCl (△) or polyethylene glycol (PEG) 400 (○) or no osmotic upshock (□). The *arrow* indicates the addition of 2,4-dinitrophenol (2 mmol/l) to the cultures and the subsequent release of [^{14}C]glycerol from cells subjected to osmotic upshock from 0.998 a_w to 0.960 a_w adjusted with NaCl (▲) or PEG 400 (●) or no osmotic upshock (■)

Table 1. The concentration of extra- and intracellular [^{14}C]glycerol 110 min after upshock of *Zygosaccharomyces rouxii* cell suspensions from 0.998 to 0.960 a_w (NaCl)

A_w	Intracellular [^{14}C]-glycerol concentration (mmol/l cell volume)[a]	Extracellular [^{14}C]-glycerol concentration (mmol/l)
0.998	2.55	10.53
0.960	26.57	6.24

[a] The cell volume was 2.23 µl/mg dry mass (van Zyl and Prior 1990)
A_w, water activity

The intracellular accumulation of the exogenously added labelled glycerol was sensitive to the energy uncoupler DNP because the addition of this compound 90 min after the initiation of the osmotic upshock with NaCl or PEG 400 from 0.998 a_w to 0.960 a_w resulted in the efflux of 83.9% and 47.4% of the accumulated extracellular labelled glycerol respectively (Fig. 1). Glycerol also leaked out of the cells upon the addition of CCCP (data not shown). These results suggest that glycerol is transported into the cell against a concentration gradient by an energy-dependent active transport system.

The influence of NaCl concentration on the accumulation of glycerol

Since no proton flux could be detected in response to the addition of glycerol, the possibility that glycerol transport is driven by the Na^+ gradient was investigated by determining glycerol accumulation during osmotic upshock from 0.998 a_w to 0.980 a_w. Various mixtures of NaCl and sucrose were used to achieve a final a_w of 0.980. Increasing NaCl concentration resulted in an exponential increase in the amount of [^{14}C]glycerol accumulated (Fig. 2) which suggested that glycerol accumulation is affected by a_w, when PEG 400 or NaCl is used to adjust the a_w (Fig. 1), as well as the NaCl concentration.

Amino acid transport by bacteria is coupled to an Na^+ gradient (Höfer 1981). The Na^+ gradient, therefore, may provide the energy for active accumulative transport of glycerol by *Z. rouxii* under salt stress but this possibility requires further investigation. Furthermore, the nature of the driving force for the accumulation of glycerol during stress imposed by osmotica other than salt is unknown. The Na^+ gradient, necessary for the accumulation of glycerol during upshock induced with an osmoticum other than NaCl, could be achieved by an Na^+/K^+ pump. The ouabain-sensitive ATPase (Na^+/K^+-activated) system is particularly important in this regard and is considered by some to be a sodium pump (Schwartz et al. 1971). An Na^+/K^+-acti-

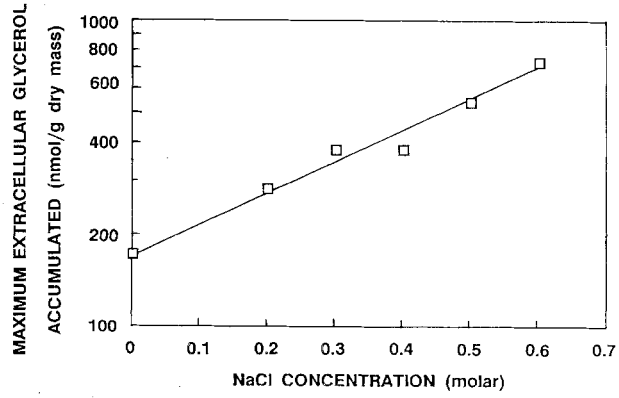

Fig. 2. The effect of osmotic upshock from 0.998 a_w to 0.980 a_w on the maximum [^{14}C]glycerol accumulated by *Z. rouxii*. Various mixtures of NaCl and sucrose were used to achieve the final a_w of 0.980

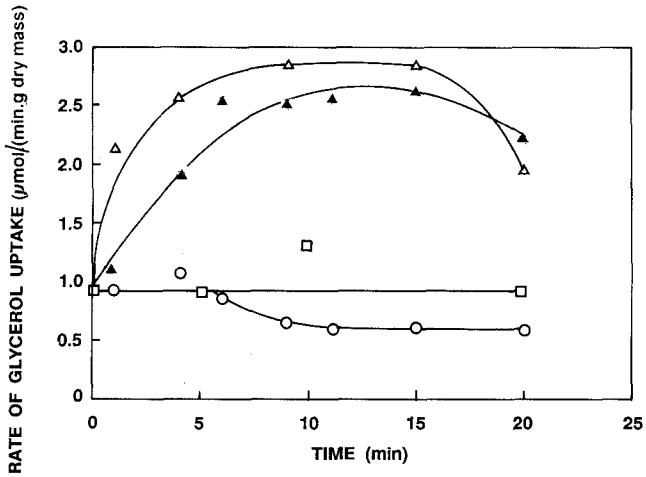

Fig. 3. The effect of either the osmotic upshock from 0.998 a_w to 0.980 a_w adjusted with NaCl (▲) or from 0.998 a_w to 0.960 a_w adjusted with NaCl (△) or PEG 400 (○) or no upshock (□) on the uptake rate of [^{14}C]glycerol by *Z. rouxii*

vated Mg^{2+}-dependent ATPase is found in *Z. rouxii* (Steinkraus et al. 1985).

The rate of glycerol uptake during osmotic upshock

The glycerol uptake rate increased from 0.9 to a maximum of 2.8 μmol/min·g dry biomass during the first 10 min of osmotic upshock from 0.998 to 0.960 a_w (NaCl) (Fig. 3). The response of the cells to the decreased a_w is too rapid for protein synthesis to be involved indicating the existence of a constitutive transport mechanism. The rate of increase in uptake rate during osmotic upshock from 0.998 a_w to 0.980 a_w (NaCl) was less pronounced than during osmotic upshock from 0.998 a_w to 0.960 a_w (NaCl). This finding supports the hypothesis that NaCl is the driving force for the accumulation of glycerol during salt stress (Fig. 3). By contrast the uptake rate during upshock from 0.998 to 0.960 a_w (PEG 400) decreased to a level lower than that of cells incubated at 0.998 a_w. Only trace elements of Na^+ are added to the growth medium in the form of Na_2MoO_4 (200 μg/l) and insufficient Na^+ may be available to provide the driving force for the accumulation of glycerol when the a_w is adjusted with PEG 400, indicating a difference in the mechanism for the accumulation of glycerol. Similarly, during osmotic upshock from 0.998 to 0.960 a_w (PEG 400), less labelled glycerol was accumulated during the same period as during osmotic upshock initiated by the addition of NaCl (Fig. 1). These differences may reflect a lower requirement for glycerol as an osmoregulatory solute when the a_w was adjusted with PEG 400. Previously we found that *Z. rouxii* accumulates greater intracellular concentrations of arabitol than glycerol during growth at decreased a_w (PEG 400) than during growth at decreased a_w (NaCl) (van Zyl and Prior 1990).

The results indicate that glycerol is actively transported into the cell by *Z. rouxii* with a carrier-mediated system with a high specificity for glycerol and that a decrease in a_w regulates the kinetics of the transport protein for glycerol. The driving force for the accumulation of glycerol by *Z. rouxii* appears to be coupled to the NaCl concentration at decreased a_w (NaCl). The mechanism of the glycerol accumulation by *Z. rouxii* in response to osmotic stress imposed by solutes other than salt is still unclear.

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